Antagonism Of Endothelin Action Normalizes Altered Levels Of VEGF And Its Signaling In The Brain Of Stroke-Prone Spontaneously Hypertensive Rat

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Abstract
Stroke-prone spontaneously hypertensive rats (SHRSP) often suffer from spontaneous stroke, in part, due to abnormalities in the cerebrovasculature. Here, we investigate the profile of key angiogenic factors and their basic signaling molecules in the brain of SHRSP during the age-dependent stages of hypertension. The profile of VEGF and its receptor, Flk-1, was dependent on age and stage of hypertension (i.e., down regulated at pre-hypertensive and malignant hypertensive stages, but up regulated at typical hypertensive stage), while that of its downstream components, pAkt and eNOS, were down regulated in a time-dependent manner in the frontal cortex of SHRSP compared to age-matched genetic control, normotensive WKY rats. On the other hand, the expression of endothelin-1 and its type A receptor (endothelin ETA receptor) were up regulated, depending on age and stage of hypertension. In contrast, levels of endothelin type B receptor were down regulated. The regional cerebral blood flow decreased during the development of malignant hypertension. Thus, subsequent experiments were designed to investigate whether endothelin-1 receptor antagonism, using endothelin-A/-B dual receptor antagonist SB209670, could normalize the molecular profile of these factors in SHRSP brain. Interestingly, blockage of endothelin-1 receptor restored to normal, levels of cerebral endothelin-1, endothelin ETA receptor and endothelin ETB receptor; VEGF and Flk-1; endothelial nitric oxide synthase (eNOS) and pAkt, in SHRSP, compared to age-matched WKY. Endothelin receptor blocker might be important to prevent the progression in the defect in VEGF and its angiogenic signaling cascade in the pathogenesis of hypertension-induced vascular remodeling in frontal cortex of SHRSP rats.

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1. Introduction

The stroke-prone spontaneously hypertensive rat (SHRSP), a substrain of SHR developed by selective breeding (Okamoto et al., 1974), is a recently characterized animal model. This model (SHRSP) has a great potential for use in studies aimed at understanding mechanisms that may underlie the pathogenesis of human cerebrovascular disorders, such as genetic hypertension, but most notably stroke, hence its name, "stroke-prone" SHR. As in humans, stroke in the SHRSP is complex, polygenic and is caused by multiple factors, but particularly by the presence of hypertension (Gratton et al., 1998; Virgintino et al., 2003). Because the SHRSP is a suitable animal model for both hypertension and stroke, it is an ideal model for studying the relationship and interaction between the two vascular disorders.

The development of hypertension in SHRSP is well established and has been divided into three consecutive stages, namely prehypertensive, typical hypertensive and malignant hypertensive, which ultimately may lead to stroke. However, the expression or profile and involvement of key vaso-regulatory factors, such as vascular endothelial growth factor (VEGF) and endothelin during the three key stages of hypertension and in different age-groups of SHRSP brain, is not known.

VEGF is an endothelial mitogen, angiogenic and potent vasopermeability factor that mediates its effects mainly through two VEGF receptor tyrosine kinases, namely fetal liver kinase 1 (Flk-1) and fms-like tyrosine kinase 1 (Flt-1) (Ferrara, 2001). Importantly, VEGF has been shown to have a diverse role in ischemic stroke, as administration of VEGF enhances angiogenesis in the ischemic brain, leading to improved neurological recovery (Zhang et al., 2000). The pattern of VEGF expression and its receptors, following occlusion of the middle cerebral artery in the rat brain, have been studied by a number of investigators (Kovacs et al., 1996; Hayashi et al., 1997; Lennmyr et al., 1988). Its been demonstrated that VEGF levels increase within the ischemic area and is localized in most cell types, such as neurons and glia, as well as infiltrating macrophages. However, because of its potent effects on vascular permeability, increased VEGF levels in the ischemic regions of the brain leads to undesirable effects, including formation of cerebral edema, increased intracranial pressure and impaired perfusion of the affected area, and ultimately to the development of infarcts (Carmeliet and Storkebaum, 2002). Moreover, intravenous infusion of recombinant human (rh) VEGF165 immediately (b 1 h) after onset of focal cerebral embolic ischemia, induce leakage of the blood-brain barrier, with resultant hemorrhagic transformation of the ischemic lesions (Zhang et al., 2000). Thus, a potential role of VEGF in different cerebral pathologies is well-documented. However, the expression or dynamics of VEGF signaling during the key stages of hypertension and in different age-groups of rats in the brain of SHRSP is unknown.

Because of the potent vasoconstrictive actions of endothelin-1 and its upregulation after stroke, this peptide has been implicated in the pathogenesis of ischemic brain lesions (Macrae et al., 1993; Patel, 1996; Lampl et al., 1997). Endothelin-1, a potent vasoconstrictor with vasoproliferative activity, is important in the development of hypertension and in target-organ damage in salt-loaded SHRSP (Okada et al., 1995; Blezer et al., 1999). A chronic blockade of the endothelin type A receptor (endothelin ETA receptor) in salt-loaded SHRSP, using a non-peptide antagonist, markedly increased survival, attenuated the development of hypertension, and prevented cerebral edema and renal damage (Blezer et al., 1999). However, as with VEGF signaling, the profile of endothelin-1 signaling during the development of hypertension and in different age-groups in the brain of SHRSP has not been clarified.

Endothelin-1 and nitric oxide (NO) are now recognized as important endothelial-derived vasomediators, the former inducing vasoconstriction and the later vasodilation (Donckier et al., 1991; Palmer et al., 1987). In addition to their role in modulating vascular tone, both could be involved in various disease processes. Of interest or importance to the present study, several studies have reported an interaction between VEGF and these two molecules (endothelin-1 and NO): 1) endothelin-1 induces levels of VEGF by increasing hypoxia-inducible factor-1α in ovarian carcinoma cells (Spinella et al., 2002), whereas in breast cancer, endothelin-1 expression has been correlated with neovascularization and high levels of VEGF (Wulfing et al., 2004), 2). The relationship between VEGF and NO is relatively better understood and is known to be context-dependent, i.e., depending on the context, NO either up regulates or down regulates VEGF expression (Kimura and Esumi, 2003). However, the relationship between endothelin-1 and VEGF in the brain tissues of animal models of genetic hypertension, such as in SHRSP, is unknown.

Such knowledge, i.e., interaction between and pattern of VEGF and endothelin-1 profile during the pre-stroke phases in SHRSP, may provide critical insights that may help elucidate mechanisms that underlie the pathogenesis of stroke. Thus, the present study was designed to investigate the profile of these two vaso-regulatory factors and their basic signaling molecules in the brain of SHRSP during the three age-dependent stages of hypertension. Secondly, we studied their potential interaction using a dual endothelin-1 receptor blocker administered from the pre-hypertensive stage.

2. Materials and methods

2.1. Animal models and treatments

The present study utilized SHRSP ($n=25$), SHR ($n=25$) (Sankyo Labo Service, Tokyo, Japan) and normotensive control WKY ($n=25$) rats aged 6, 20 and 40 weeks old. SHRSP and normotensive rats were originally donated by the late Prof Kozo Okamoto, Department of Pathology, Kinki University School of Medicine, Osaka, Japan, and inbred in our laboratory (current generation F57). All animals were housed in a room maintained at temperature, 22±2 °C; relative humidity, 55±10%: and 12-h light/dark cycle (lights on at 6:00 AM). The animals were allowed free access to food and water. The experimental design was approved by the Animal Care and Use Committees of Hokkaido University School of Medicine and Tsukuba University.

Following the various treatments described below, the rats were killed by exsanguinations under a lethal anaesthetic dose of ketamine, and the whole brain was rapidly excised and immediately frozen in liquid nitrogen and stored at −80 °C for later use.

2.2. Treatments

Group 1: the blood pressure of SHRSP, SHR and WKY rats aged 6, 20, 40 weeks was measured using the tail-cuff method. The rats were then killed and their frontal cortices processed, as described above.

Group 2: in another set of experiments, 6-week old SHRSP rats were either given an endothelin dual receptor antagonist, SB209670 [(+)-(1S,2R,3S)-3-(2-carboxymethoxy4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-

carboxylic acid] (n=15) (1 mg/day: SmithKline Beecham Pharmaceuticals, PA, U.S.A.), or saline (n=15) (vehicle), subcutaneously using an osmotic pump (Model 2ML4, DURECT Corporation, Cupertino, CA, U.S.A.), implanted in the back. The rats were treated for 12 weeks and examined at 18 weeks, a time when they were in the typical hypertensive stage. Control rats, WKY rats (n=15), were inserted with osmotic pumps containing saline.

Group 3: in another setting, some control WKY rats were treated with dual endothelin receptor antagonist, SB209670, as described for group 2 above (n=6 for each group).

2.3. Monitoring of regional cerebral blood flow

Rats were anesthetized with 1% halothane in 20% O_2/N_2, and concentric guide cannulae were stereotactically implanted to the surface of the duramater of the right prefrontal cortex (3.2 mm anterior and 0.7 mm lateral to the bregma), according to the stereotaxic co-ordinates. Two days after implantation, a fiber-optic probe with a diameter of 0.5 mm (Omegawave, Tokyo, Japan) was inserted via the guide cannula, and regional cerebral blood flow was measured using a laser-Doppler flowmetry (Omegaflow FLO-C1; Omegawave, Tokyo, Japan), under freely moving conditions (Jesmin et al., 2004). The average of the value obtained every 0.5 s for 5 min (600 points) was evaluated as a basal regional cerebral blood flow.

2.4. Enzyme immunoassay for endothelin-1

The concentrations of endothelin-1 in plasma and extracts of the frontal cortex of both treated and untreated rats were determined using an Endothelin-1 Enzyme Immuno Assay Kit (Immuno-Biological Laboratories, Fujioka, Japan). The reported cross-reactivity of the antibody for the former was b0.1% for all the endothelins, b0.1% for endothelin-3, and 3.3% for endothelin-2.

2.5. Western blot analysis

Changes in levels of the proteins of interest were determined by immunoblotting, as demonstrated in our previous report (Jesmin et al., 2004). Briefly, samples of tissue homogenate were subjected to electrophoresis on polyacrylamide gels, and proteins were transferred to polyvinylidine difloride filter membrane. After blocking with 5% non-fat milk in phosphate-buffered saline, the membranes were incubated with specific antibodies recognizing either VEGF (Immunological Laboratories, Fujioka, Japan), Flk-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated Akt (pAkt) (Cell Signaling Technology, Beverly, MA, USA), endothelial nitric oxide synthase (eNOS) (Affinity BioReagents, Golden, CT, USA), endothelin ETA receptor (Alomone Labs, Jerusalem, Israel), or endothelin ETB receptor (Alomone Labs) antibodies. Two antibodies for VEGF, namely mouse anti-human VEGF antibody (Santa Cruz Biotechnology) and anti-human VEGF rabbit polyclonal antibody (Immunobiological Laboratories), were used in the present study to detect VEGF by immunoblot analysis. These antibodies recognize both the 165 and 121 amino acid residue forms of VEGF and were able to recognize the target peptide in rats. In most cases, the specificity of each antibody was confirmed in preliminary studies by blocking their immunoreactivity using specific competing peptides against which the antibodies were raised. It should also be noted that immunoreactivity disappeared when non-immune IgG was used instead of the primary antibodies. After extensive washing with phosphate-buffered saline containing 0.1% Tween 20, designed to remove any nonspecifically bound primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody. Negligible loading/transfer variation was observed between samples. Moreover, β-Actin was used as a loading control (anti-

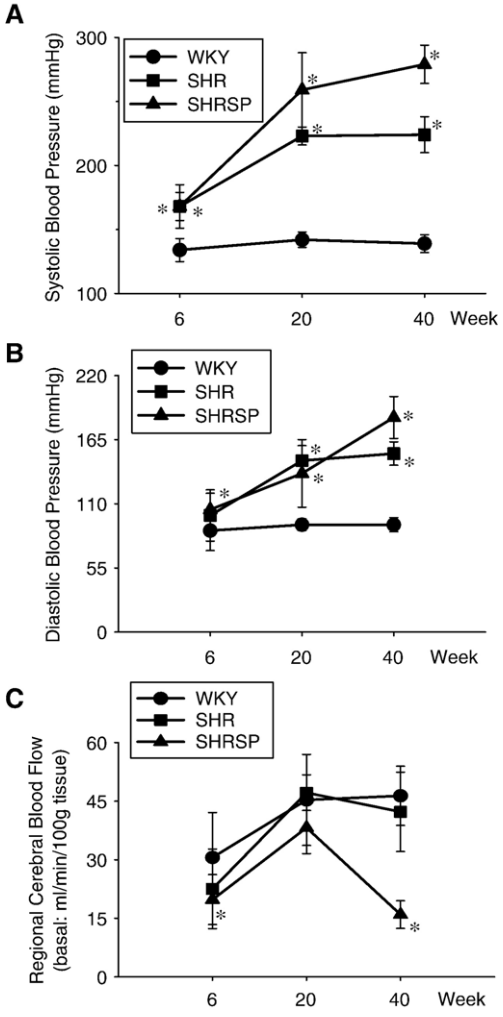

Fig. 1. (A) Systolic blood pressure (n=20 for each group), (B), diastolic blood pressure (n=20 for each group) and (C) measurement of regional cerebral blood flow in the frontal cortex of WKY (n=5), SHRSP (n=5) and SHR (n=5) from 6 to 40 weeks of age. Regional cerebral blood flow has been expressed as ml/min/100 g tissue. Data are means±S.D. *pb0.05 compared with the corresponding values obtained in WKY at each respective age.

Xenopus laevis β-Actin mouse monoclonal antibody, Abcam). The blots were visualized with the enhanced chemiluminescence detection system (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK), exposed to X-ray film, and analyzed by NIH image software.

2.6. RNA preparation and real-time quantitative PCR

Real-time quantitative PCR was performed, according to the protocols and primer sequences described in our recent report (Jesmin et al., 2004).

2.7. Capillary morphology

Previous studies have shown that histochemical staining with the lectin *Griffonia simplicifolia* (GSA-B4) is a sensitive and reliable method to visualize the capillary vasculature in the frontal cortex of rat brain (Jesmin et al., 2003). Eight-micrometer-thick serial frozen coronal sections of frontal cortex were stained with GSA-B4 (Sigma Chemical, St. Louis, MO). The sections were fixed in acetone, air dried, and placed in PBS. After treatment with 3% H_2O_2 in methanol and washing in PBS, the sections were incubated with GSA-B4

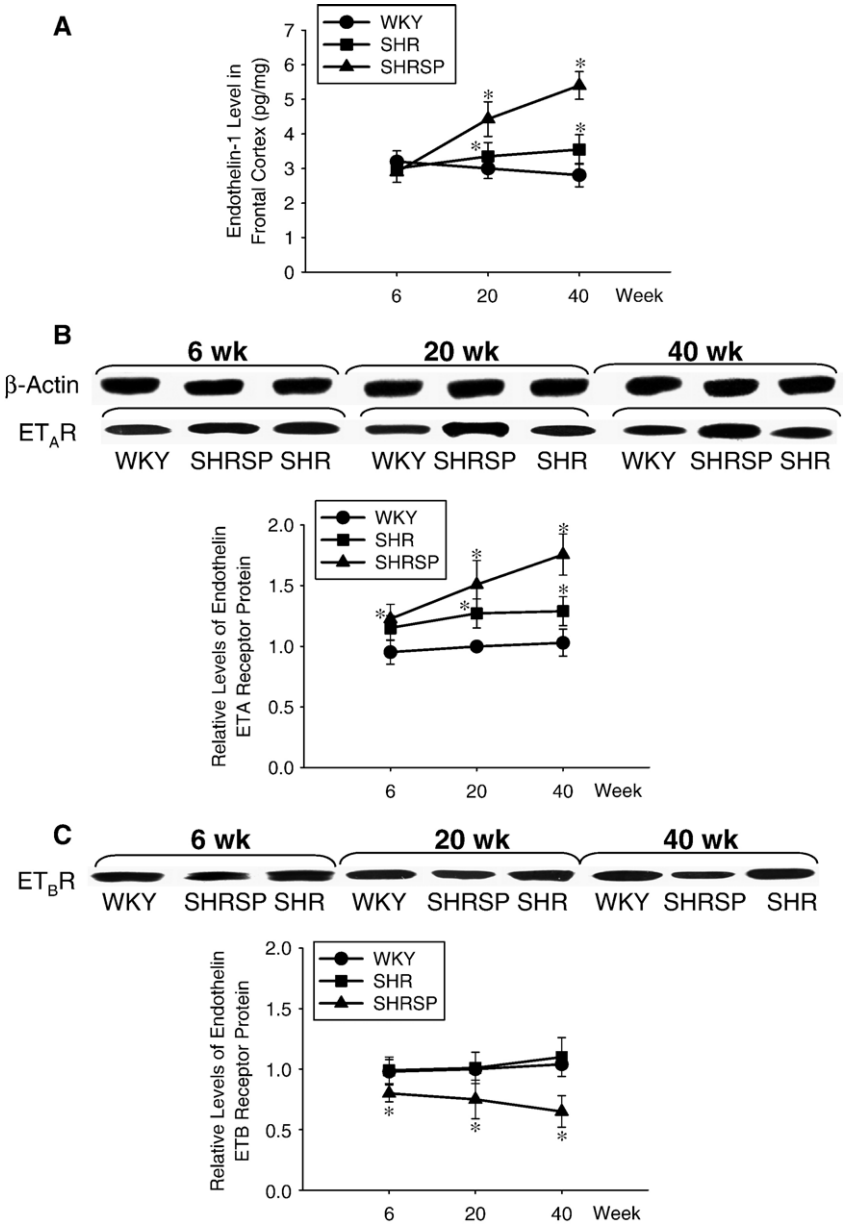

Fig. 2. (A) Endothelin-1 levels in frontocortical tissues of WKY, SHRSP and SHR from 6 to 40 weeks of age. Tissue endothelin-1 level was determined by ELISA. (B, C), Immunoblot analyses for endothelin ETA receptor and endothelin ETB receptor, respectively, in the frontocortical tissues of WKY, SHRSP and SHR rats from 6 to 40 weeks of age with representative blots and densitometries, respectively. The experiments were conducted by loading equal amounts of WKY, SHRSP and SHR frontocortical proteins in each lane. β-Actin was used as loading control. The protein level of each band obtained in 20-week-old WKY is normalized as 1.0. Values are means± S.D. of 8 preparations from different animals in each group. *pb0.01 vs. age-matched WKY.

(1:100 dilution in PBS) overnight at 4 °C. This was immediately followed by reaction with streptoavidin conjugated to peroxidase (Nichirei Corp., Tokyo, Japan), rinsing in PBS and visualization using diaminobenzidine/H_2O_2 as a chromogen. To enhance the diaminobenzidine reaction, the sections were rinsed with 0.05 M $NaHCO_3$ (pH 9.6) and then incubated in diaminobenzidine enhancing solution (Vector Laboratories, Burlingame, CA). Vascular endothelium was stained with lectin, which stained capillaries as black/dark brown dots. Sections were examined using a microscope (Olympus, Tokyo, Japan), and counts were made of stained capillaries in cross-sections in 30 fields (117,617 μm^2/field) per sample (section on slide) at a final magnification of ×400 by an image-analyzing software (microcomputer imaging device, Imaging Research, St. Catharine, Ontario, Canada). This counting method has been well established by previous reports (Jesmin et al., 2003, 2002a,b). Furthermore, the number of lectin-stained capillaries were quantified by two independent researchers in a double-blinded study. Care was taken to avoid counting the same single capillary twice. Any microvessel (defined as a vessel having internal diameter b100 μm) that had no apparent lumen was considered as a single capillary.

2.8. Immunohistochemical studies

Immunohistochemical labeling of frontocortical tissues were performed, as described in our previous study (Jesmin et al., 2003, 2002a,b). Briefly, after tissues were cut (5 μm thick), the sections were deparaffinized and treated for 20 min with citrate buffer (10 mM citric acid, pH 6.0) in a microwave oven (750 W) before immunostaining. In some cases, frozen sections were fixed in acetone and air dried. After blocking non-specific staining from secondary antibodies, the sections were incubated with primary antibodies overnight at 4 °C, followed by incubation with appropriate secondary antibodies coupled to horseradish peroxidase. The immunostaining findings were then viewed by light microscopy by incubating tissue sections with AEC (3-amino-9-ethylcarbazole) Peroxidase Substrate Solution.

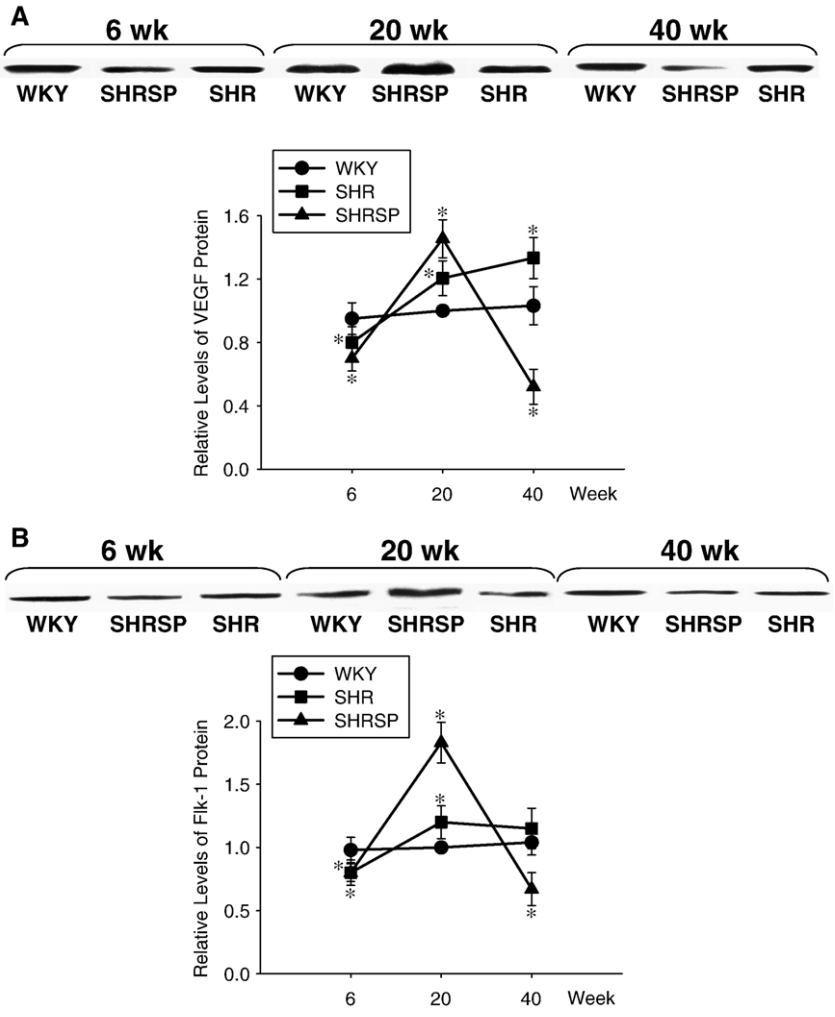

Fig. 3. Representative Western blots of VEGF (A) and Flk-1 (B) with densitometric analyses in frontocortical tissues from WKY, SHR, and SHRSP from 6 to 40 weeks of age. The protein level of each band obtained in 20-week-old WKY is normalized as 1.0. Values are means±S.D. of 8 preparations from different animals in each group. *pb0.01 vs. age-matched WKY.

2.9. Statistical analysis

The values are expressed as means±S.D. Statistical analysis of the data was performed by one way analysis of variance (ANOVA), followed by Fisher's protected least significance *t*-test and Scheffé's test to determine the significance of the differences in multiple comparisons. A *p*-value less than 0.05 was considered significant.

3. Results

Part 1 summarizes data demonstrating age-dependent alterations in blood pressure, cerebral blood flow, and signaling transductions of VEGF and endothelin-1.

3.1. Blood pressure changes

Systolic blood pressure of SHRSP increased with age, reaching 276±14 mmHg (n=20) at 40 weeks of age (Fig. 1A). At 6 weeks of age, the blood pressure of SHRSP was already higher than that of WKY (168±17 *vs.* 132±8 mmHg, pb0.05). Although the systolic blood pressure of SHR was consistently found to be significantly higher than that of WKY, hypertension in SHR was established at the age of 20 weeks (Fig. 1A). The diastolic blood pressure was always significantly higher in SHRSP rats at all the ages examined than in WKY rats (Fig. 1B).

3.2. Measurements of regional cerebral blood flow

A significant decrease of regional cerebral blood flow was observed in SHRSP frontal cortex at 6 weeks and 35-40 weeks of age, compared to age-matched WKY. At the typical hypertensive stage (18-20 weeks of age) in SHRSP, regional cerebral blood flow tended to decrease, although it was statistically insignificant compared to age-matched WKY. However, changes in regional cerebral blood flow of SHR were insignificant at all the ages compared to age-matched WKY (Fig. 1C).

3.3. Endothelin-1 level and expression of endothelin receptors

Endothelin-1 levels in the frontal cortex of both SHRSP and SHR increased in a time-dependent manner compared to age-

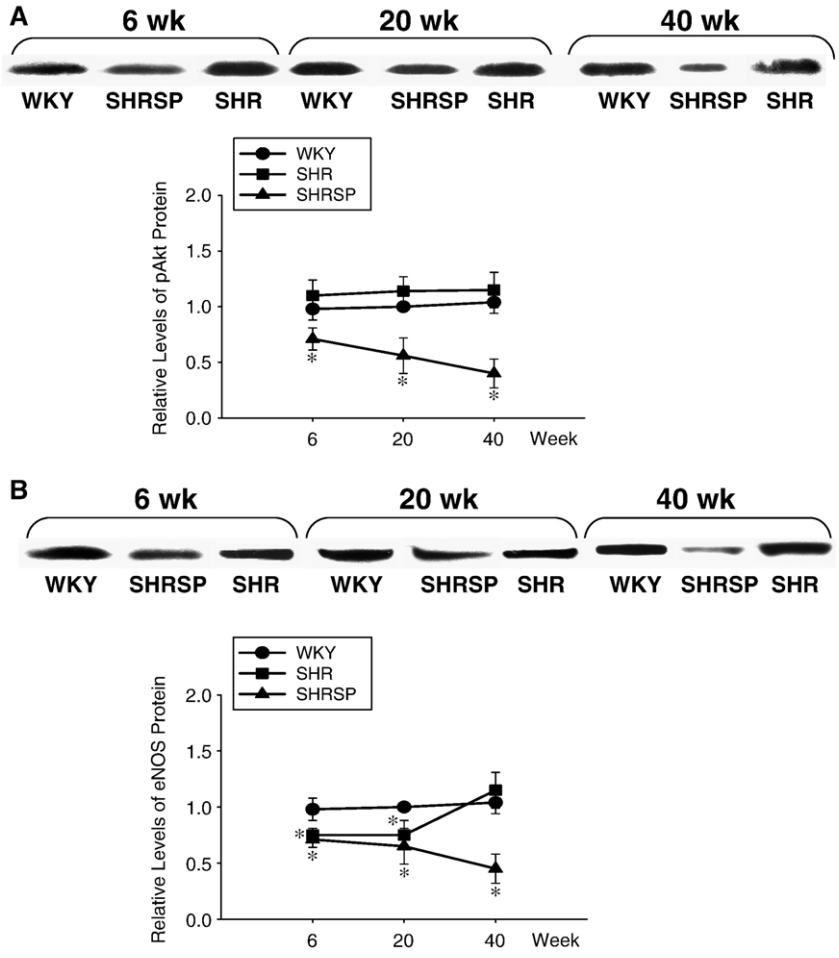

Fig. 4. Representative Western blots of pAkt (A) and eNOS (B) with densitometric analyses in frontocortical tissues from WKY, SHR, and SHRSP from 6 to 40 weeks of age. The protein level of each band obtained in 20-week-old WKY is normalized as 1.0. Values are means±S.D. of 8 preparations from different animals in each group. *pb0.01 vs. age-matched WKY.

matched WKY (Fig. 2A). However, the extent of the increase was greater in SHRSP than in SHR (Fig. 2A).

Levels of endothelin ETA receptor were up regulated in SHRSP frontal cortex, along with its peptide ligand, endothelin-1, from the prehypertensive stage to malignant hypertensive stage, compared to age-matched WKY (Fig. 2B). In contrast, those of endothelin ETB receptor were down regulated in a time-dependent manner (Fig. 2C). The expression patterns of endothelin receptors were different in SHR frontal cortex from those of SHRSP (Fig. 2).

3.4. Expression of VEGF and Flk-1

Levels of VEGF and Flk-1 in the frontocortical tissues of WKY, SHR, and SHRSP for all ages, as demonstrated by the typical immunoblots and the cumulative data for quantitative immunoblotting, are shown in Fig. 3A and B, respectively. Expression of VEGF and Flk-1 in WKY was not affected by age, whereas in SHRSP, there was a pronounced increase, relative to WKY, between 6 to 20 weeks of age. However, the expression pattern in SHR was different from SHRSP. Between 35-40 weeks of age, levels of VEGF and Flk-1 in the frontal cortex of SHRSP decreased by 50% relative to WKY (Fig. 3A and B). The protein expressions for VEGF and Flk-1 were consistent with mRNA levels obtained from real-time PCR (data not shown).

3.5. Expression of pAkt and eNOS

Typical immunoblots of pAkt and eNOS in the frontocortex of WKY, SHR, and SHRSP, at all ages examined, and the cumulative data for quantitative immunoblotting, are shown in Fig. 4A and B, respectively. In SHRSP, pAkt and eNOS expressions were significantly less than in WKY between 6 to

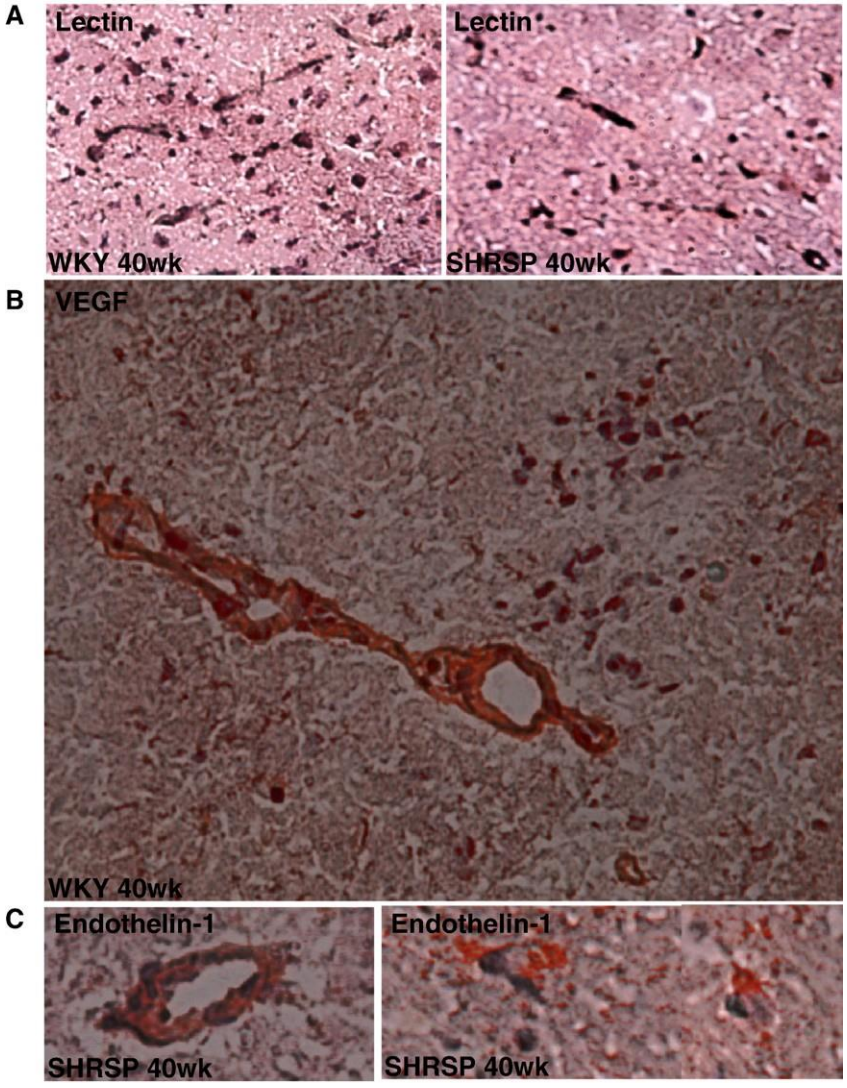

Fig. 5. (A), Morphology of cerebral capillaries in frontal cortex sections from WKYand SHRSP at 40 weeks of age. Photomicrographs of frontal cortex sections stained with lectin appearing as black/dark brown dots are shown. Magnification ×400. (B), Representative immunohistochemical image of VEGF (red color) in frontocortical tissues from WKY at 40 weeks of age. (C), Representative immunohistochemical image of endothelin-1 (red color) in frontocortical tissues from SHRSP at 40 weeks of age. Magnification ×400.

40 weeks of age, with a marked decrease between 20 and 40 weeks of age (Fig. 4A and B).

3.6. Capillary morphology

Capillary density in the frontal cortex of 40 week-old SHRSP rats, as determined by GS4 lectin staining, was significantly lower (43%) compared to age-matched genetic control WKYs ($p<0.001$) (Fig. 5A). At the typical hypertensive stage (18-20 weeks old), the capillary density in frontal cortex of SHRSP rats was slightly decreased (17%) compared to age-matched WKY rats ($p<0.01$).

3.7. Cellular localization of Endothelin-1 and VEGF system

The expression of endothelin-1, endothelin receptors, VEGF and the components of its angiogenic signaling transduction (Flk-1, pAkt and eNOS) were expressed abundantly in both the

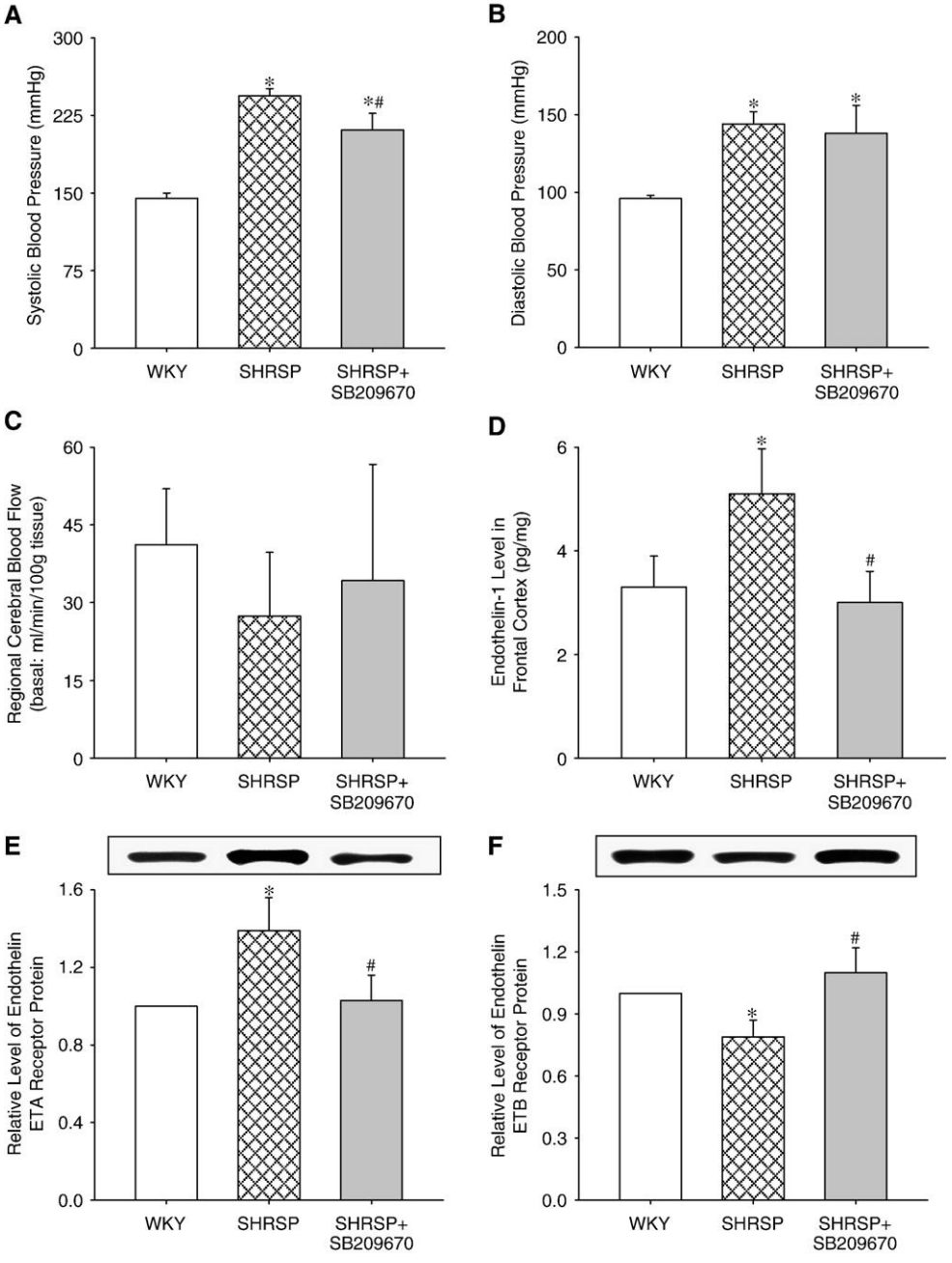

Fig. 6. Effects of endothelin receptor antagonist on (A) systolic blood pressure, (B) diastolic blood pressure, (C) regional cerebral blood flow, and frontocortical (D) endothelin-1 levels by ELISA in WKY, vehicle-treated SHRSP (SHRSP), and endothelin receptor antagonist-treated SHRSP (SHRSP+ SB209670) rats. Protein expressions of endothelin ETA receptor (E) and endothelin ETB receptor (F) in the frontocortical tissues from WKY, vehicle-treated SHRSP (SHRSP), and endothelin receptor antagonist-treated SHRSP (SHRSP+ SB209670) rats. The panel of bands, just above the histogram, shows representative blots of the type of animal, as described above. In each of the experiments, the band obtained from WKY is normalized as 1.0. Data are shown as means±S.D. of seven separate experiments. *$p<0.01$ vs. age-matched WKY, #$p<0.01$ vs. vehicle-treated SHRSP.

vascular and neuronal tissues of the frontal cortex (Fig. 5B and C). Moreover, in some cases, the expression was also localized in glial cells.

Part 2 summarizes the effects of endothelin receptor antagonism on different end-points or parameters investigated in SHRSP rats.

3.8. Blood pressure

After endothelin receptor antagonism, although the decrease in systolic blood pressure of SHRSP rats appeared to be slight, it was statistically significant (pb0.05) (Fig. 6A). However, the diastolic blood pressure remained unchanged (SHRSP rats) after endothelin receptor antagonism (WKY: SHRSP: SHRSP+209670: 96±2: 144±8: 138±18 mmHg) (Fig. 6B).

3.9. Regional cerebral blood flow

Although the regional CBF at 20 weeks of age in the frontal cortex of SHRSP tended to decrease, as shown previously in Fig. 1C, it was statistically insignificant. After endothelin receptor antagonism, the regional cerebral blood flow showed an apparent improvement, which was also statistically insignificant (Fig. 6C).

3.10. Endothelin-1 level and expression of endothelin receptors

Endothelin receptor antagonist normalized altered (up regulated) levels of endothelin-1 in the frontal cortex of SHRSP (Fig. 6D), as well as that of endothelin receptors, after a 12-week treatment (Fig. 6E and F). The protein expressions of endothelin receptors were consistent with mRNA levels (data not shown).

3.11. Expression of VEGF and Flk-1

The three months treatments with dual endothelin receptor blocker, starting from 6 weeks of age (pre-hypertensive stage) normalized levels of VEGF and Flk-1 in frontal cortex of SHRSP compared to those of WKY (Fig. 7A and B).

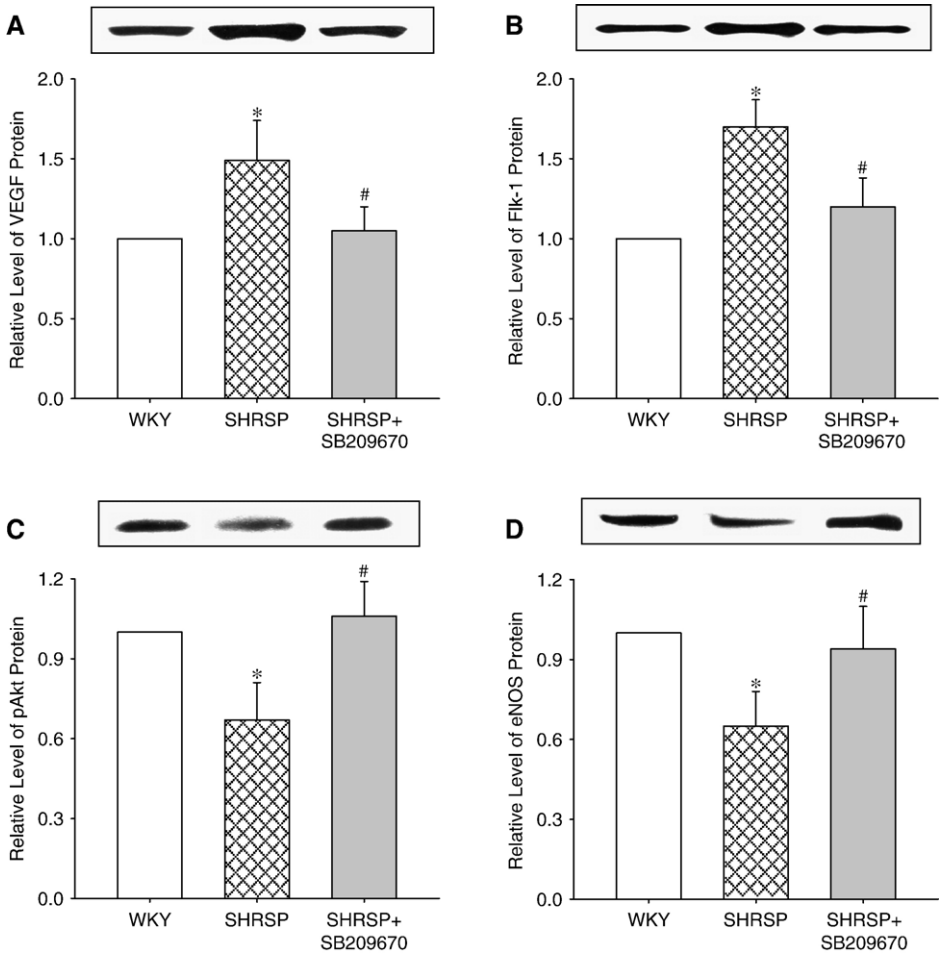

Fig. 7. Protein expressions of VEGF (A), Flk-1 (B), pAkt (C) and eNOS (D) in the frontocortical tissues from WKY, vehicle-treated SHRSP (SHRSP), and endothelin receptor antagonist-treated SHRSP (SHRSP+ SB209670) rats. The panel of bands, just above the histogram, shows representative blots of the type of animal, as described above. In each of the experiments, the band obtained from control WKY is normalized as 1.0. Data are shown as means±S.D. of seven separate experiments. *pb0.01 vs. age-matched WKY, #pb0.01 vs. vehicle-treated SHRSP.

3.12. Expression of pAkt and eNOS

Long-term treatment with dual endothelin receptor blocker normalized the immunoreactivity of pAkt and eNOS in frontal cortex in SHRSP compared to those in WKY (Fig. 7C and D).

3.13. Capillary morphology

The slight decrease in the capillary density of the frontal cortex in SHRSP rats was ameliorated by endothelin receptor antagonist.

Part 3 summarizes the effects of endothelin receptor antagonism on different parameters in WKY rats. When control WKY rats were treated with SB209670, both the systolic and diastolic blood pressure tended to decrease. However, this change was statistically insignificant (Fig. 8A and B). Treatment of WKY rats with SB209670 also did not have any effect on the cerebral expression of endothelin receptors and components of VEGF signaling (Fig. 8).

4. Discussion

One of the major findings of the present study is the demonstration that levels of VEGF and its angiogenic receptor, Flk-1, are altered age-dependently in the frontal cortex of SHRSP, i.e., they decreased slightly during pre-hypertension (6 weeks); significantly increased during the typical hypertension (10-20 weeks); and markedly decreased during the malignant hypertension (35-40 weeks). On the other hand, levels of endothelin-1 and endothelin ETA receptor, were up-regulated during the three stages of hypertension, while

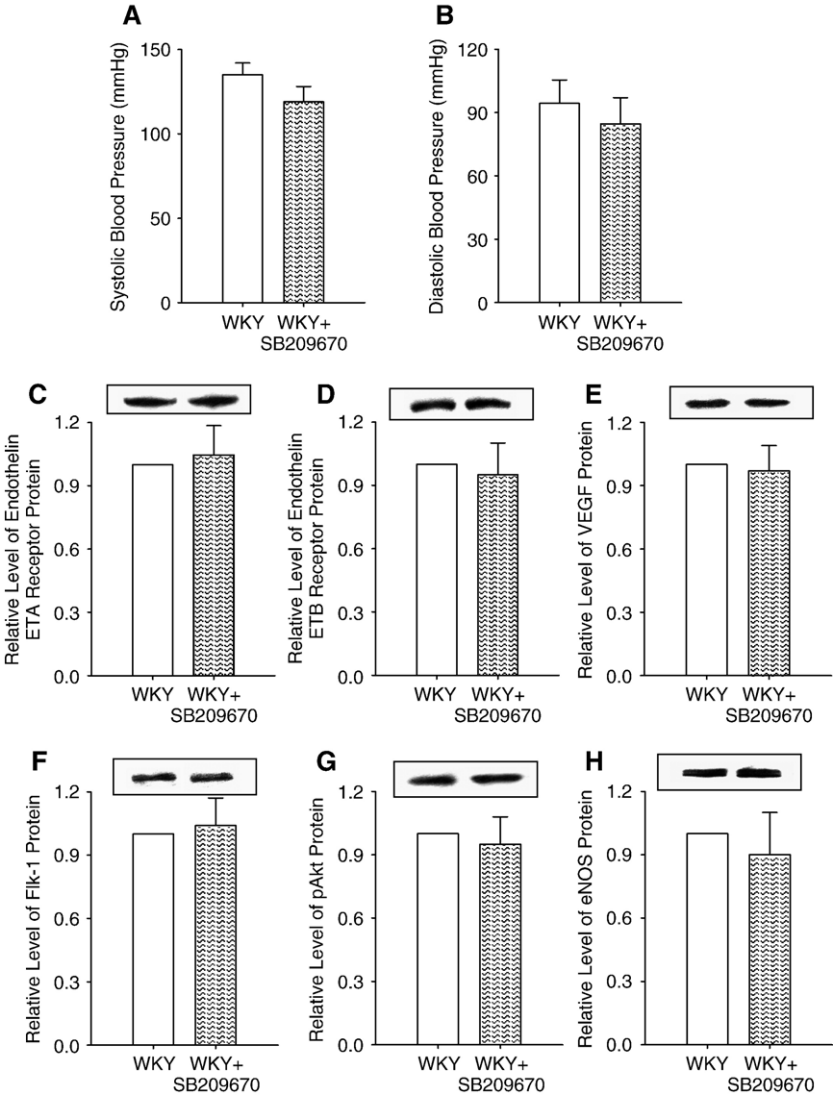

Fig. 8. Effects of endothelin receptor antagonist on (A) systolic blood pressure, (B) diastolic blood pressure, frontocortical protein expression of (C) endothelin ETA receptor, (D) endothelin ETB receptor, (E) VEGF, (F) Flk-1, (G) pAkt and (H) eNOS in vehicle-treated WKY (WKY), and endothelin receptor antagonist-treated WKY (WKY+SB209670) rats. The panel of bands, just above the histogram, shows representative blots of the type of animal, as described above. In each of the experiments, the band obtained from WKY is normalized as 1.0. Data are shown as means±S.D. of six separate experiments.

endothelin ETB receptor was down regulated in a time-dependent manner. Moreover, there was a decline in frontal cortical blood flow at the typical hypertensive stage, which became remarkably significant at malignant hypertension. Further, the capillary density in the frontal cortex of SHRSP brain was also remarkably lower compared to the age-matched WKY rats at malignant hypertension. Of interest, endothelin receptor antagonism for three months, starting from the prehypertensive stage, significantly normalized levels of both VEGF and endothelin-1 signaling components compared to WKY control and slightly lowered the systolic blood pressure, without altering diastolic blood pressure in SHRSP rats. In contrast, endothelin receptor antagonism in WKY rats failed to reduce both the systolic and diastolic blood pressure significantly.

Because SHRSP and SHR are nearly equally hypertensive at typical hypertensive stage, the differences in the profiles of angiogenic factors (VEGF and its signaling molecules) are unlikely to be due to elevated blood pressure. Levels of VEGF, along with its signaling molecules, are likely to be altered in abnormal regional cerebral blood flow (Jesmin et al., 2004). Because Akt plays a role in diverse cellular processes that contribute to the angiogenic process, its status within endothelial cells is critical for blood vessel growth (Kureishi et al., 2000; Shiojima and Walsh, 2002). The importance of eNOS in VEGF signaling is well documented (Ziche et al., 1997; Paxian et al., 2004). Impaired angiogenesis in eNOS-knockout mice is not improved by giving VEGF (Kimura and Esumi, 2003) and *in vitro* studies have shown that VEGF-induced endothelial cell migration and proliferation can be significantly depressed by inhibition of eNOS action (Shizukuda et al., 1999; Murohara et al., 1999). Thus, Akt and eNOS are essential mediators in the angiogenic signaling cascade of VEGF. The simultaneous reduction in VEGF-signaling, regional cerebral blood flow and capillary density during the malignant hypertensive stage, a phase that precede stroke, may potentially create conditions that favor the pathogenesis of stroke in this animal model. To the best of our knowledge, the present study provides the first report showing a time-dependent alteration of VEGF signaling pathway and regional cerebral blood flow in the frontocortex of SHRSP.

Factors likely to underlie hypertension include endothelial damage and abnormality in angiogenesis, which correlate with the overall cardiovascular and cerebrovascular risks (Felmeden et al., 2003). Another concept of impaired vascular development suggests structural alterations of microvascular beds, such as capillary rarefaction. Because angiogenic growth factors, are major regulators of blood vessel formation, such abnormal changes in angiogenesis are ultimately associated with altered expressions of these markers (angiogenic factors) (Hutchins and Darnell, 1994; Sullivan et al., 1983; Virgintino et al., 2003). For instance, evidence of angiogenesis in full-blown atherosclerosis includes increased numbers of vasa vasorum in arteries burdened with atheroma, which are associated with increased expression of angiogenic growth factors. Moreover, recent data demonstrated an increase in plasma levels of VEGF in hypertensive patients, suggesting that the impaired capacity for vascular growth may be related to abnormal regulation of VEGF (Felmeden et al., 2003). Here, we show that although VEGF and its angiogenic receptor are upregulated in SHRSP brain at typical hypertensive stage, the cerebral blood flow did not increase, but rather tended to decrease. Therefore, we speculate that there might be abnormal regulation of VEGF signaling, as well as abnormal vascular growth in the SHRSP brain, making it susceptible to stroke. Thus, it is crucial to arrest the defect in VEGF angiogenic signaling in frontal cortex prior to the development of malignant hypertension in SHRSP rats.

The present finding shows an association between up-regulated levels of endothelin-1 and endothelin ETA receptor and vasoconstriction in SHRSP brain. It is of interest that this association was observed beginning from the pre-hypertensive-malignant stages, consistent with an earlier report that showed increased endothelin-1 binding in SHRSP brain (Savage and Jeng, 2002). In contrast to endothelin ETA receptor, endothelin ETB receptor, which mediates the vasodilatory properties of endothelin-1 (Seo et al., 1994), was down-regulated in SHRSP brain in an age-related manner. Thus, we argue that the augmented expression of endothelin-1 and endothelin ETA receptor in the frontal cortex of SHRSP contributes to various cerebrovascular remodeling that compromise blood flow volume. When endothelin ETA receptor causes vasoconstriction, endothelin ETB receptor activation induces endothelium-dependent relaxation through the release of NO and prostacyclin (Seo et al., 1994). The balance between vasoconstriction and vasorelaxation or endothelin ETA receptor and endothelin ETB receptor is the most important factor in determining the regional blood flow and blood pressure regulatory effects of endothelin-1 (Schiffrin, 2001). This conclusion is consistent with a report by Armstead (Armstead, 2003) which stated that endothelin-1 concentration is elevated in cerebrospinal fluid and contributes to the impaired cerebral hemodynamics following fluid percussion brain injury (FPI) in an age-dependent manner. Thus, we here suggest that suppression of VEGF angiogenic signaling cascade and the associated up regulation of endothelin-1/endothelin ETA receptor in the frontal cortex of SHRSP rat, coupled with the development of malignant hypertension, may contribute to decreased regional cerebral blood flow and capillary microvasculature. Ultimately, this may lead to stroke. The therapeutic implications of these findings are that, at least in SHRSP rats, early reversal or correction of altered levels of VEGF and endothelin signaling is critical when performed prior to a significant drop in regional cerebral blood flow.

In the second part of this study, we treated SHRSP rats with dual endothelin receptor antagonist for three months, starting from the prehypertensive stage, and found that endothelin antagonism greatly normalized the altered levels of VEGF and endothelin signaling in SHRSP brain. The normalization of these critical vasoregulatory pathways occurs at a stage when regional cerebral blood flow was not yet significantly compromised, although a slight decrease in capillary density in frontal cortex was observed. Conflicting results exists on the interaction between VEGF and endothelin-1. Since its been shown that endothelin-1 induces VEGF mRNA expression through the endothelin ETA receptor in vascular smooth muscle

cells (Pedram et al., 1997) and that endothelin-1 is able to induce angiogenic responses in cultured endothelial cells and stimulates neovascularization *in vivo* in concert with VEGF (Salani et al., 2000), we speculate that their altered relationship and interaction may contribute to the pathogenesis of the three stages of hypertension in SHRSP. If such a prediction is true, blockage of one of the two factors should normalize levels of either one or both VEGF and endothelin-1, and their associated molecules. Interestingly, consistent with our speculation, the signaling of both molecules was normalized by endothelin receptor antagonism. The exact mechanisms and/or relationship between the two angiogenic factors are currently unclear. It is possible that endothelin-1 alters levels of VEGF via mRNA transcription in vascular cells (Matsuura et al., 1998), and that increase in levels of endothelin-1 alters VEGF signaling and, subsequently, regional cerebral blood flow in SHRSP brain. Moreover, the interaction among the different components of endothelin system is complex and depends on a variety of factors. For instance, after endothelin receptor antagonism, levels of the unbound endothelin-1 seem to increase, even though those of the cerebral tissues and plasma are suppressed. In addition, endothelin receptor antagonism also reverses the alterations in endothelin receptors in frontal cortex of SHRSP rats. While complex feedback regulatory mechanisms persist among different components of endothelin system, at the same time other vasoregulatory molecules, such as nitric oxide, may also play a role in the regulation of endothelin-1 (Lavallee and Thorin, 2003; Schiffrin, 2001).

The mechanisms that underlie the reversal effects of the dual endothelin receptor blocker on the various target molecules investigated here, may be non-hemodynamic since changes in the blood pressure in both SHRSP and WKY rats were not so different. Moreover, blockade of endothelin receptor in WKY rats did not alter either the components of VEGF signaling or endothelin receptors in the frontal cortex. Thus, it is reasonable to state that the effects of endothelin receptor blockade observed in SHRSP brain, is likely a specific event in SHRSP rats. However, we cannot at this point, based on the data obtained, delineate the specific mechanism that underlies the normalization or reversal of altered VEGF and endothelin-1 signaling by endothelin receptor antagonism in the SHRSP brain. Reversal of altered VEGF signaling cascade, accompanied by reduced cerebral endothelin-1, indicate that autocrine/paracrine endothelin-1 secretion pathways could be a factor in the neurovascular remodeling blunted by endothelin receptor antagonist. Decreases in regional endothelin-1 concentrations may also result from decreases in endothelin-1 production and/or increased endothelin-1 clearance. The production of endothelin-1 begins with the cleavage of the translational product preproendothelin-1 (Lavallee and Thorin, 2003; Schiffrin, 2001) and our results show inhibition of endothelin receptor antagonist on preproendothelin-1 mRNA at the transcription levels (data not shown). Thus, decrease in endothelin-1 production may play a major role in regional endothelin-1 changes. Together, both secretion and synthesis of endothelin-1 may be inhibited by endothelin receptor antagonism, leading to reduced levels of endothelin-1 and normalization of altered VEGF signaling cascade. Moreover, it is our assumption that the blockade of endothelin ETA receptor may decrease levels of endothelin-1 due to the suppression of the positive feedback mechanism between endothelin-1 and endothelin ETA receptor. Enhanced endothelin-1 level has been shown to decrease endothelin ETB receptor activation and levels (Black et al., 1998). Thus, after endothelin receptor blockade, the cerebral endothelin ETB receptor level is normalized, likely due to the withdrawal effect of the inhibitory responses of the elevated endothelin-1 on cerebral endothelin ETB receptor expression. Endothelin-1 has been also shown to down regulate eNOS expression through endothelin ETA receptor (Wedgwood and Black, 2005). Thus, the decreased level of endothelin-1 by the dual receptor blocker might help in the recovery of eNOS expression in SHRSP brain. Indeed, coupling between endothelin ETB receptor stimulation and eNOS activation decreases sinusoidal constriction and plays a functionally important role in maintaining microcirculation and tissue oxygenation in the normal liver (Paxian et al., 2004). eNOS activity is increased only with endothelin ETA receptor plus endothelin ETB receptor antagonist, not by endothelin ETA receptor antagonist only, suggesting that endothelin ETB receptor is associated with posttranscriptional regulation of NOS in hypercholesterolemia (Taner et al., 2001). Moreover, age-related dysregulation of nitric oxide-dependent mechanisms may also impact endothelin-1 responses (Besse et al., 2001; Lavallee and Thorin, 2003; Tschudi and Luscher, 1995). Because nitric oxide (NO) may exert important inhibitory effects on endothelin-1 mediated signaling in some vascular beds (Lavallee and Thorin, 2003), we speculated that aberrations in the functional cross-talk between endothelin receptors and NO might converge to exacerbate age-related and hypertension-dependent enhancements in endothelin-1-mediated cerebral constrictor responses. However, future studies will aim to elucidate potential significant interactive effects between NO and endothelin receptor signal transduction pathways and their effects on the vascular dysregulation associated with aging and the progression of hypertension.

The present study used a dual endothelin receptor blocker to see the changes in different target molecules in SHRSP brain. To further consolidate the hypothesis of the present investigation, future studies should use specific endothelin receptor antagonist. Moreover, the measurement of regional cerebral blood flow was done in only four (n=4) SHRSP rats after endothelin receptor antagonism. In order to arrive at a more convincing conclusion, the sample size of SHRSP rats should be increased and data obtained evaluated for regional cerebral blood flow after endothelin receptor antagonism. Lastly, the effect of endothelin receptor blocker in SHRSP rats should be performed prior to the time point when regional cerebral blood flow begins to significantly decline.

In conclusion, SHRSP exhibits an age-dependent alteration in the signaling of VEGF in the frontal cortex. This alteration was accompanied by reduction in regional cerebral blood flow and up-regulation of endothelin-1 and endothelin ETA receptor. Administration of the dual endothelin receptor blocker normalized levels of both VEGF and endothelin-1, with their associated molecules.

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